Curr Microbiol (2015) 70:470–475
DOI 10.1007/s00284-014-0746-x

First Study of Different Insect Cells to Triatoma Virus Infection

María Laura Susevich · Gerardo Aníbal Marti · Germán Ernesto Metz · María Gabriela Echeverría

Received: 26 August 2014 / Accepted: 23 October 2014 / Published online: 7 December 2014

Abstract The use of viruses for biological control is a new option to be considered. The family *Dicistroviridae*, which affects only invertebrates, is one of the families that have been proposed for this purpose. The Triatoma virus (TrV), a member of this family, affects triatomine transmitters of Chagas disease, which is endemic in Latin America but also expanding its worldwide distribution. To this end, we attempted virus replication in Diptera, *Aedes albopictus* (clone C6/36) and Lepidoptera *Spodoptera frugiperda* (SF9, SF21) and High Five (H5) cell lines. The methodologies used were transfection process, direct inoculation (purified virus), and inoculation of purified virus with trypsin. Results were confirmed by SDS-PAGE, Western blotting, RT-PCR, electron microscopy, and immunofluorescence. According to the results obtained, further analysis of susceptibility/infection of H5 cells to TrV required to be studied.

Introduction

The Triatoma virus (TrV) is the only entomopathogenic virus found and identified in triatomines [17]. TrV is a member of the family *Dicistroviridae*, whose type species is the cricket paralysis virus (CrPV), a single-stranded RNA virus consisting of 9,010 bases that replicates in intestinal epithelial cells, causing delayed development and death of infected insects [16]. The TrV genome has two open reading frames, ORF1 and ORF2, with 5,387 and 2,606 nucleotides, respectively. These ORFs are separated by an intergenic region of 172 nucleotides (nt). ORF1 is located between nt 549 and 5,936 and codes for non-structural proteins, whereas ORF2 is located between nt 6,109 and 8,715 [8] and codes for the structural proteins of the viral capsid: VP2, VP4, VP3, and VP1 [1, 2]. Due to its high pathogenicity and vertical transmission, TrV is considered a potential agent for biological control of its host *Triatoma infestans* [16], the vector of the protozoan parasite *Trypanosoma cruzi*, which causes Chagas disease in humans. About seven to eight million people are estimated to be infected with this disease in Latin America [23]. Consequently, TrV has the potential to be exploited to control its disease-bearing hosts [9]. Viruses are associated with insects in a wide range of ecological relationships: as pathogens, vectors, or symbionts [7]. There are at least 14 families with representative viral pathogens of invertebrates and some of these families have viruses that have been investigated as biological control agents of insects. For that reason, in 2002, TrV was assigned to the family *Dicistroviridae*, genus Cripavirus, together with eight species: Drosophila C virus (DCV), Aphid lethal paralysis virus (ALPV), Cricket paralysis virus (CrPV), Black queen cell virus (BQCV), Himetobi P virus (HiPV), Plautia stali intestine virus (PSIV), *Rhopalosiphum padi* virus (RhPV), and *Homolodisca coagulata* virus (HoCV-1). A second genus named Aparavirus was recently included, which comprises six species: Acute bee paralysis virus (ABPV), Taura syndrome virus (TSV), Kashmir bee virus (KBV),

M. L. Susevich · G. A. Marti
Centro de Estudios Parasitológicos y de Vectores (CEPAVE-CCT-La Plata-CONICET-UNLP), 2 #584, 1900 La Plata, Argentina

M. L. Susevich · G. A. Marti · G. E. Metz · M. G. Echeverría
CONICET (CCT-La Plata), La Plata, Argentina

G. E. Metz · M. G. Echeverría (✉)
Virology, Faculty of Veterinary Sciences, National University of La Plata, La Plata, Argentina
e-mail: gecheverria@fcv.unlp.edu.ar

Solenopsis invicta virus-1 (SINV-1), Israeli acute paralysis virus (IAPV), and Mud crub virus (McV) [11]. In the USA, because the invasion of the red imported fire ant (*Solenopsis invicta*) and the glassy-winged sharpshooter (*Homalodisca coagulata*) has resulted in high costs because of the loss of animals, the large losses in vineyards and the treatment of affected victims, the use of dicistroviruses as biopesticides is being considered [3].

CrPV can be taken as a model because it belongs to the same family and genus as TrV. CrPV has a wide host range and easily replicates in different insect cell lines. This virus easily adapts to growing in suspension cultures in a large scale, such as cells of Diptera (DL2) [21], and causes cytopathic effect in some cell lines derived from lepidoptera [15]. Using purified viral particles and viral RNA transfection, Masoumi et al. 2003, further described that CrPV is able to absorb and replicate in seven cell lines, with the appearance of cytopathic effect [14]. Regarding DCV, it has been found that it easily replicates in many cell lines derived from Drosophila [6]. Regarding RhPV, using viral RNA transfection, Boyapalle et al. 2007, evaluated its replication in nine cell lines derived from lepidoptera, diptera, and hemiptera and found that from the nine cell lines used, only two (Hemiptera) were permissive for RhPV with appearance of cytopathic effect [4].

Until now, no cell line has been reported to be susceptible to TrV. In addition, it is not known whether this virus causes some cytopathic effect on the infected cells. The aim of our work was to assess whether the virus is able to infect and replicate in various cell lines to rule out the possibility of cross infection associated with wildlife or other beneficial insects.

Materials and Methods

Source of TrV

Viral particles were purified from insects obtained from a colony experimentally infected with TrV (by feeding upon virus-contaminated substrata) of *T. infestans,* maintained at the Centro de Estudios Parasitológicos y de Vectores (CEPAVE), La Plata, Argentina. A total of 30 infected adult insects were dissected, and their midguts were homogenized in 10 ml of NMT buffer (0.01 M NaCl, 0.001 M $MgCl_2$ and 0.04 M Tris–HCl, pH 7.4). The homogenate was clarified and centrifuged at 140,000×*g* for 3 h at 4 °C to obtain TrV particles. The pellet was then resuspended in NMT buffer and layered on top of a continuous sucrose gradient (10–30 %, w/v). After centrifugation at 64,000×*g* for 3 h at 4 °C, 2-ml fractions were obtained using a peristaltic pump. The selected fractions, measured at 260 nm, were diluted in NMT buffer and then centrifuged at 44,000×*g* for 2 h at 4 °C. This final pellet was resuspended in 1 ml TE buffer (Tris–EDTA) (1 mM EDTA, 10 mM Tris–HCl, pH 7.4). Then, the protein concentration of the fractions was determined by the Bradford total protein content assay using a Bio Rad protein assay kit with bovine serumalbumin (BSA) as the standard. Besides, each fraction was subjected to 12.5 % polyacrylamide gels using the discontinous system (SDS-PAGE) [13]. The virus was stored at −70 °C until use. In order to demonstrate virus viability, an inoculation of TrV intrahemocelically was performed in the triatomines insectary from CEPAVE (free from TrV insects). As other authors we found the death of triatomines after 72 h post inoculation [16].

Cell Culture

Cell lines of diptera, *Aedes albopictus* (clone C6/36) and lepidoptera *Spodoptera frugiperda* (SF9, SF21) and High Five (H5) were grown and maintained in either minimal essential medium (MEM) C6/36 or TC-100 medium with fetal calf serum (FCS) (10 or 2 %) in cell culture flasks of 25 cm^3 (Greiner Bio-One BioScience, USA) and 75 cm^3 (Greiner Bio-One BioScience, USA).

RNA Extraction and Purification

Viral RNA was extracted from purified TrV by conventional methodology phenol/guanidine thiocyanate (Trizol Invitrogen). Then, 500 µl of purified virus was mixed with 500 µl of Trizol and 220 µl of chloroform. After mixing and centrifugation, the upper aqueous phase was transferred to a new tube and precipitation was performed with 750 µl of isopropanol overnight at −70 °C. After centrifugation, the pellet was washed three times with 200 µl of ethanol and after drying, RNA was resuspended in 20 µl of Nuclease-free water. Finally, the concentration was determined by measuring absorbance at 260 nm in a spectrophotometer [4, 14].

Transfection Process

Each cell line was seeded into six-well or twelve-well culture plates at a density of 1–2 × 10^5 cells per well and allowed to attach to the plate for 4 h at 28° C. The non-adherent cells and medium were removed and replaced with serum-free medium, and each well was treated with the mixtures consisting of 2 or 5 µl RNA or 5 µl RNA + lipofectine according to the manufacturer's instructions (Invitrogen). All mixtures were incubated at room temperature for 45 min. Subsequently, each mixture was filled with 800 µl TC-100 medium and then added to each well and plates were incubated for 4 h at 28 °C. Finally, 1 ml of TC-100 with 2 % FCS was added to each

well and observed for 7 days. After that, two blind passages were made in each fresh cell line every 7 days [4, 14].

RNA Electroporation

For RNA electroporation, 10 µl of a concentration of approximately 277 ng/µl of RNA was used for each assay. One T-25 flask of each cell line was washed twice and resuspended in 1 ml of medium and put in an electroporation chamber with 10 µl of RNA. Another flask of each cell line was used as control. Electroporation was performed on ice at 310 V with a single pulse (2.8 ms, 750 V/cm, 10 uf), and then placed on ice for 10 min. Then, the cells were seeded in six-well plates. At 4 h of incubation at 28 °C, the medium was removed and replaced with fresh medium with 2 % FCS and observed for 7 days. After 1 week, two blind passages were made in each fresh cell line.

Direct Inoculation: Purified Virus

Approximately 10–20 µg of purified virus in 0.5 ml of phosphate buffered saline (PBS), previously filtered through a 0.22 µm filter, was used for each assay [15]. Each cell line was seeded into three wells of a 12-well culture plate. A volume of 0.2 ml of each cell line was inoculated, and one well of each cell line was left as control. Plates were incubated for 1 h at 28 °C and the plates gently moved every 15 min. Then, 1 ml of maintenance medium was added. Cells were incubated at 28 °C for 72–96 h. After 1 week, four blind passages were made in each fresh cell line every 7 days.

Inoculation of Purified Virus with Trypsin

Inoculation of purified virus with trypsin is used routinely in many naked viruses to facilitate viral penetration. Medium for infection was prepared with 0.5 µg/ml of trypsin [10]. Cells were observed daily to assess the cytopathic effect (CPE) and after 1 week, two blind passages were made in each cell type every 7 days.

Confirmation Techniques

To evaluate the presence of TrV in the cells by different methods, five techniques were used: SDS-PAGE, Western blotting (WB), and RT-PCR as described by Marti et al. 2008 [13]; electron microscopic examinations using supernatant and cell pellets as described by Rozas-Dennis and Cazzaniga 2000 [20] and indirect immunofluorescence (IIF). IIF was performed in confluent monolayers of SF9, SF21, H5, and C6/36 cells grown in culture chambers (BD Falcon). Cells were inoculated with supernatants (50 µl) from different passages of transfection, electroporation, and purified virus with or without trypsin and incubated for 1 h at 28 °C and then completed with TC-100 medium with 2 % FCS (200 µl). After 72 h, the supernatant was collected and cells were fixed with acetone at −20 °C overnight. Slides were washed three times with PBS and then incubated with rabbit anti-TrV serum at different dilutions (1/20, 1/40, 1/80) for 45 min at 37 °C. After three washes with PBS, goat anti-rabbit FITC-conjugated serum diluted 1/100 and 1/200 was added and incubated for 45 min at 37 °C in a humid chamber. Finally, after five washes, the chamber was mounted (50 % glycerol + 50 % PBS) and observed in an Olympus fluorescence microscope.

Results and Discussion

Figure 1 displayed a SDS-PAGE of TrV after sucrose gradient purification. The concentration of purified virus selected for the experiences was 0.2 mg/ml. After Trizol extraction from purified virus, 277 ng/µl TrV RNA was obtained. The inoculation of TrV in insect cell culture by different methods, purified particles or purified RNA only in H5 cells showed a visible CPE within the first 24–48 h. The cells became bigger and rounded and approximately 10 % of cells were floating. Besides, cells revealed a granular appearance. After successive passages, this CPE disappeared. All these experiments were performed twice.

By SDS-PAGE of cellular pellets, a positive result was observed in H5 cells by TrV direct inoculation and negative results after successive passages (Fig. 2). On the other hand, by WB, an unspecific positive result was obtained after the first passage, using electroporation in C6/36 and SF9 cells, given a single band that appeared also in control cells. RT-PCR gave a positive result only after the first

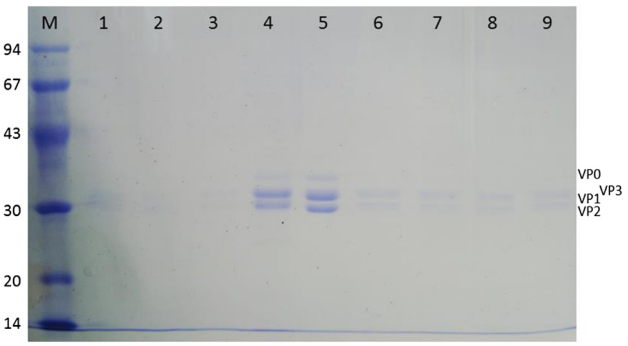

Fig. 1 12.5 % SDS-PAGE of purified Triatoma virus obtained after sucrose gradient and stained with Coomassie blue. *M* molecular weight marker; *lanes* 1–9 different gradient fractions of TrV; *lane* 5 was choose for the experiences; on the *right*: TrV structural proteins

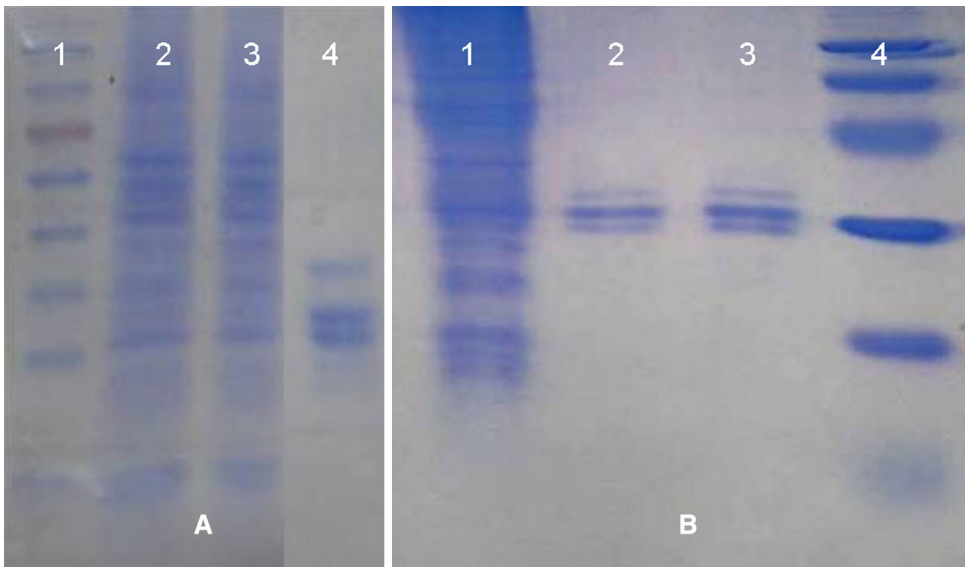

Fig. 2 12.5 % SDS-PAGE. **a** 1 Page ruler prestained protein ladder; 2 SF9 mock-infected cells. 3 SF9 cells infected with purified TrV; 4 TrV control. **b** 1 H5 cells infected with purified TrV; 2 and 3 TrV control; 4 low marker

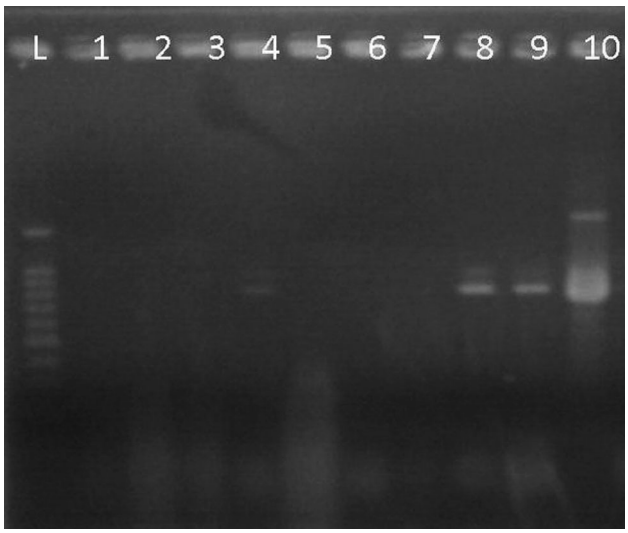

Fig. 3 RT-PCR. *L* 100-bp ladder; 1–3 control cells (C6/36, SF9, H5); 4–6 cells transfected with TrV RNA using lipofectin (C6/36; SF9; H5); 7–9 cells infected with purified TrV particles (C6/36; SF9; H5). 10: TrV positive control

passage in C6/36, SF9, and H5 cells (Fig. 3). By electron microscope, no TrV particles were observed in mock-infected cells (Fig. 4). By IIF, cells showed unspecific fluorescence.

In this study, we assayed different confirmatory tests to analyze evidence of replication of TrV in different cultured cell lines. We found a positive result for detection of viral RNA by RT-PCR but only in the first passage in three cultured cell lines. The amount of RNA used in our transfection technique by lipofectin was according to that previously reported by others [4, 14] (1–4 μg), whereas the amount of purified TrV was according to that previously reported by Moore et al. 1980 [15]. Nevertheless, this result was not detected after successive cell passages. This finding indicated the absence of viral replication and the concomitant loss of viral progeny. Thus, the positive results obtained may be due to the presence of virus remained from the inoculum, as was found by others [19]. These results showed that all cell lines studied were not permissive to TrV replication because we were unable to detect RNA or viral proteins after successive cell passages. In contrast, we hypothesized that H5 cell line where a positive result was found might be subject to a deep analysis, assuming that this cells are susceptible to viral infection but they are not permissive to viral replication.

Few tests have been performed on *Dicistroviriidae* family members using different insect cell lines. In the case of CrPV, Scotti et al. 1996, found that *Trichoplusia ni* (TN368) cells produce more particles than *Drosophila* line 2 (DL2) cells. These authors attributed these differences to a different metabolism or different susceptibility between Lepidoptera (TN368) and Diptera (DL2) cell lines. They also reported the propagation of CrPV in cell suspension [21]. Christian and Scotti 1996, showed the ability of CrPV to replicate and observed CPE in cell lines derived from Lepidoptera and Diptera. This virus replicates well in *Anticarsia gemmatalis*, *T. ni* and *Spodopthera ornithogalli* (Lepidoptera), and DL2 (Diptera) but shows poor replication in *Helicoverpa zea* (Lepidoptera) and *Aedes albopictus* (Diptera). These authors pointed out that replication usually coincide with the presence of CPE in infected cells. Only in the case of *Cydia pomonella* (Lepidoptera), there is considerable replication in the absence of any detectable CPE [5, 6].

Masoumi et al. 2003, had excellent results both with CrPV purified virions and RNA transfection in DL2, *Plutella xylostella,* and *A. gemmatalis,* and although not all

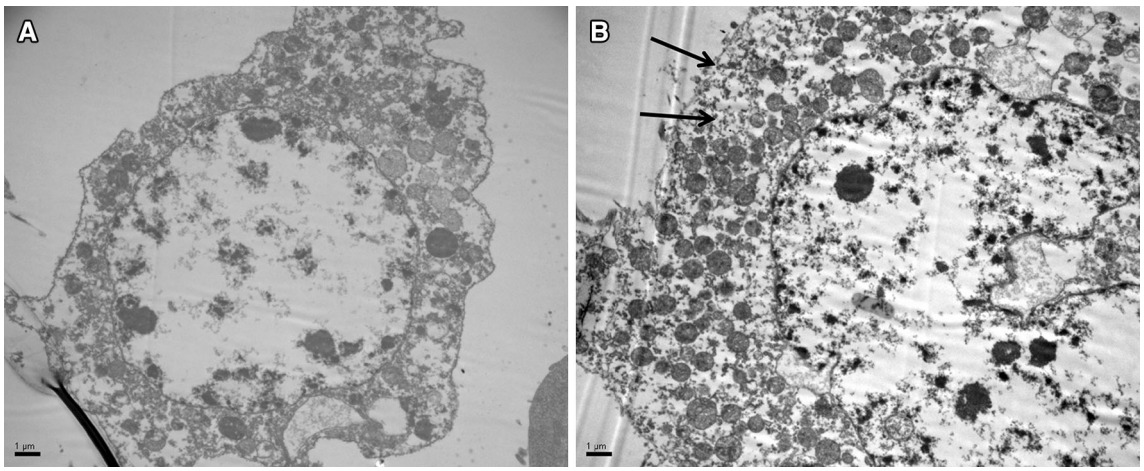

Fig. 4 Electronic microphotographs. **a** H5 control cells (×8,000); **b** H5 cells infected with purified TrV (×10,000). *Arrows* indicate electron dense structures similar to TrV virus particles

showed CPE, only DL2 showed marked CPE using both viral particles and RNA transfection [14]. In view of the differences in the dissimilar ability of insect cells to allow viral replication, these authors postulated the hypothesis that the permissibility of CrPV to cell lines is not exclusively determined by the functionality of CrPV IRES (Internal Ribosome Entry Site); the intact IRES is a prerequisite for other viral functions but is not sufficient by itself to ensure viral replication. Cellular factors and/or viral factors are needed to process and assemble mature virions [14].

In the case of RhPV, another family member of *Dicistroviridae*, Boyapalle et al. 2007, identified a cell line (Hemiptera) that allowed its replication, which had not hitherto been possible to replicate in cell lines. This means that cellular factors are present to process the viral genome and assemble viral particles; therefore, complete infectious particles morphologically indistinguishable from purified virus are produced. This shows that although both RhPV and TrV belong to the same family, different cells respond to the viral infection, and even the CPE could be presented in the first four or up to 10 days after infection according to the cell type and number of viral particles [4].

Within dicistroviruses, the Taura syndrome virus (TSV) is an important virus prevalent in shrimp farming areas and its primary host, *Litopenaeus vannamei*, is consumed by people around the world [18]. In China, some people eat fresh shrimp without cleaning; however, there is no evidence that TSV can infect humans. Luo et al. 2004 showed that this virus cannot infect mammalian cell lines or insect cell lines [12]. So, although no positive results have been obtained by others for TSV [19], we will attempt to replicate TrV in mammalian cell lines. For these reasons, because TrV has so far failed to replicate in Diptera and Lepidoptera cells, and thinking that it could be used in the future as a biological control agent, our next search will be guided to know if this virus could be a potential pathogen to Hemiptera as well as to mammalian cell lines.

In the present study, we did not obtain repeatability of positive results after the first passage. According to our results, TrV does not replicate in cell lines from Lepidoptera and Diptera. This is the first report of the intent of TrV replication in these insect cell lines. Until now, no cell line has been reported to be susceptible to TrV. High Five cell line is a possible candidate to a further study because of the positive results obtained in this work. Although we did not discard to study other cell lines as possible candidates for viral replication. Consequently, the further study of TrV–cell interaction will be an important tool to select a cell line candidate to obtain viral progeny [22].

Acknowledgments This study was partially funded by Grants PIP 0007 (CONICET) and PICT 2011-1081 (ANPCyT), Argentina. We thank the Cátedra de Virología, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Argentina, and CEPAVE (Centro de Estudios Parasitológicos y de Vectores) where this work was developed.

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